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PRETREATMENT OF HUMAN EPIDERMAL KERATINOCYTES WITH D,L-SULFORAPHANE PROTECTS AGAINST SULFUR MUSTARD CYTOTOXICITY

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Sulfur mustard (SM) is a powerful cytotoxic agent as well as a potent vesicant, mutagen, and carcinogen. This compound reacts with glutathione (GSH) and forms GSH-SM conjugates that appear to be excreted through the mercapturic acid pathway in mammals. The question of whether glutathione-S-transferases (GST) are involved in enzymatic formation of these conjugates remains unresolved. In previous studies, ethacrynic acid (EAA), a putative inhibitor of this transferase, and oltipraz, a known inducer,were ineffective in modulating this enzyme in cultured normal human epidermal keratinocytes (NHEK) so this hypothesis could not be tested. Higher levels of intracellular GSH appeared to be solely responsible for resistance of EAA-pretreated cells to SM. A better inducer of GST was needed to test whether this enzyme could be used to modify cytotoxicity following SM exposure. D,L-sulforaphane (DLS), a compound from broccoli extract known to be a potent inducer of this enzyme, was tested for GST induction in cultured NHEK. The enzyme levels increased optimally (40%) in these cells within 4 hours using 0.5 μg DLS/mL over a 48 hour incubation period. When the drug was removed by washing, and pretreated cells were challenged with 0–200 μM SM, there was a 10%–15% increase in survival at 24 hours compared with non-pretreated SM controls. This protective effect due to increased levels of GST was abolished at 300 μM sulfur mustard, where there was no difference in survival between pretreated and non-pretreated controls. Glutathione levels were also assessed and showed no increase at 4 hours in cultured NHEK with DLS pretreatment and appear not to be responsible for this protection against SM.

Keywords: Cytotoxicity; D,L-sulforaphane; Pretreatment; Sulfur mustard

INTRODUCTION

Sulfur mustard (SM) is a chemical warfare agent that is best known for its potent vesicant (blister-forming) ability in skin, but it also causes debilitating respiratory and ocular lesions in individuals without protective masks. There is no proven medical countermeasure that can interfere with its pathology despite intensive research efforts in many laboratories throughout the world since World War I (WWI) (1).

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Models that truly mimic human skin and form frank blisters are nonexistent, so alternative methods use *in vitro* models for research on the specific areas of interest. In our laboratories, normal human epidermal keratinocytes (NHEK) are used as the *in vitro* model (2) for skin because of several advantages such as (a) applicability as a target cell of SM exposure; (b) relative low cost compared with other approaches; and (c) ease of studying biochemical mechanisms present in the epidermal layer, which is not easily accomplished with intact skin. In this system, biochemical pathways amenable to medical intervention can be studied in an efficient and convenient fashion. Also, variables such as cell density and growth stage can also be carefully controlled, allowing research during specific metabolic phases of the cell.

Studies have shown that some of the related electrophilic nitrogen mustards used in cancer chemotherapy become less efficacious by reacting with glutathione (GSH) and forming conjugates (3). The rationale for this study was based on this observation, which proposed a mechanism for sulfur mustard intervention by forming conjugates between SM and GSH in our *in vitro* model. These conjugates can form spontaneously or through a Phase II detoxication enzyme mediated process that involves the catalytic formation of the GSH-SM conjugate.

In these studies, NHEK were pretreated for up to 48 hours using D,L-Sulforaphane (DLS) [a selective inducer of Phase II enzymes (4) such as GST in other systems], and the intracellular level of this enzyme was measured. The conditions necessary to optimize enzyme levels were determined, and the NHEK were challenged with SM after removal of the DLS. The viabilities of the pretreated cells and non-pretreated cells were measured 24 hours later (5) and compared to determine whether pretreatment had any beneficial effect. These studies may be helpful in deciding whether GST could be involved in modifying SM-induced cytotoxicity.

MATERIALS AND METHODS

Materials

Normal human epidermal keratinocytes (NHEK), Keratinocyte Growth Media (KGM), and Trypsin-EDTA Reagent Packs were purchased from Cambrex Bioscience (Walkersville, MD). Tissue culture vessels were purchased from Corning Corporation (Corning, NY) or Falcon Corporation (Newark, NJ). The Cell Titer 96 AQueous® Non-radioactive Cell Proliferation kits, which measure the conversion of the unique tetrazolium compound [3-(4,5-dimethyl thiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] to a yellowish water-soluble formazan in the presence of an electron coupling reagent, phenazine methosulfate (PMS), by dehydrogenase enzymes, were purchased from Promega (Madison, WI). D,L-sulforaphane (DLS), 5-sulfosalicylic acid (SSA), and other laboratory chemicals were purchased from Sigma Chemical Company (St. Louis, MO). The Misonix® tissue culture plate sonicator was purchased from Misonix® Corporation (Farmingdale, NY).

Cell Culture

The NHEK were purchased as second passage cells and cultured in KGM in T-75 flasks in a 5% CO_2 incubator at 37°C. They were subsequently subcultured into

24-well plates at the desired density. Cells were grown until estimated confluencies of 60%–80% were reached prior to experimentation.

Drug Addition

The DLS was dissolved in DMSO at 25 mg/mL and aliquots were frozen at −80°C until usage. The DLS was thawed and diluted appropriately into fresh KGM at concentrations ranging from 0.1–10 μg/mL of KGM. Cells were incubated in a 5% CO_2 incubator at 37°C for up to 48 hours. The drug was removed by 3 cycles of washing with saline, and the plates were either frozen at −80°C for later analysis or replenished with fresh KGM for further experimentation.

Exposure of NHEK to SM

A stock solution of 4 mM SM was diluted into KGM and added to the appropriate cell culture plates in a chemical surety hood to yield final SM concentrations of 10, 50, 100, 200, and 300 μM. After 1 hour at ambient temperature to allow reaction and hydrolysis of agent, the tissue culture plates were transferred to a 37°C incubator under a humidified 5% CO_2 atmosphere for postexposure incubation. Removal of SM is unnecessary since earlier studies showed that it is hydrolyzed to non-cytotoxic products (6), which are considered innocuous.

Disruption of NHEK

Thawed NHEK were disrupted by adding 300 μL of 0.1 M KPO_4 buffer, pH 6.5 to each well of the plate. The plate was covered with an acetate film and sonicated for four 30 second cycles with 1 minute of cooling at 4°C between cycles on a Misonix® plate sonicator. Lysis appeared complete by microscopy and the supernatant fluid was assayed for GSH and GST levels as described later.

GSH Analysis of NHEK

The supernatant liquid was withdrawn in 15 μL aliquots and then analyzed for total GSH (reduced and oxidized forms) by the GSH reductase cycling assay (7). Positive controls for GSH liberation in NHEK were established by extracting NHEK with 300 μL of 5% SSA. Analysis of 15 μL aliquots of the acid-extracted NHEK was accomplished by the same cycling assay after the appropriate dilution.

GST Analysis of NHEK

The supernatant (100 μL) from disrupted NHEK was added to 100 μL of an assay mixture containing 0.5 mM chlorodinitrobenzene (CDNB), 1 mM GSH, and 100 mM KPO_4 buffer, pH 6.5 in a 96-well plate, and the absorbance at 340 nm was measured kinetically in a microplate scanning spectrophotometer (8).

Assessment of NHEK viability in 24-well Plates

Cell viability in 24-well plates was determined using MTS as an indicator of mitochondrial dehydrogenase activity, which forms a water-soluble formazan from MTS that is measured spectrophotometrically at 490 nm. A 500 μL portion of the supernatant media was aspirated and discarded from each well; an aliquot of 50 μL MTS-PMS solution was added to each well, and the plates were incubated at 37°C in a 5% CO_2 incubator for an additional 4 hours. Samples of 100 μL from each well (in duplicate) were then transferred to a clean 96-well plate with wells containing 150 μL H_2O and read at 490 nm using a microplate scanning spectrophotometer. Blank values were subtracted and viability was calculated as the percent of control viability.

Statistical Analysis

Data were analyzed by *t*-test and the significance level was $p < 0.05$ (*) when compared with untreated controls.

RESULTS

When NHEK were incubated with the various concentrations of DLS shown in Fig. 1, the levels of GST remained constant for 2 hours. There is an approximate 40%–50% increase in GST levels at 4 hours that disappears after 8 hours. At later

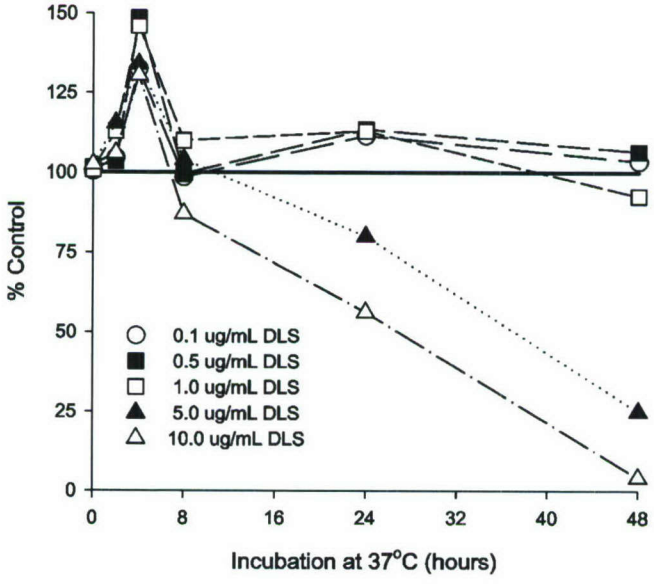

Figure 1 The effect of DLS on GST levels in NHEK. The NHEK were incubated for up to 48 hours in 5% CO_2 at 37°C with the indicated concentrations of DLS. The drug was removed and GST was measured as described in Materials and Methods. Each point represents the average of triplicate samples from two experiments.

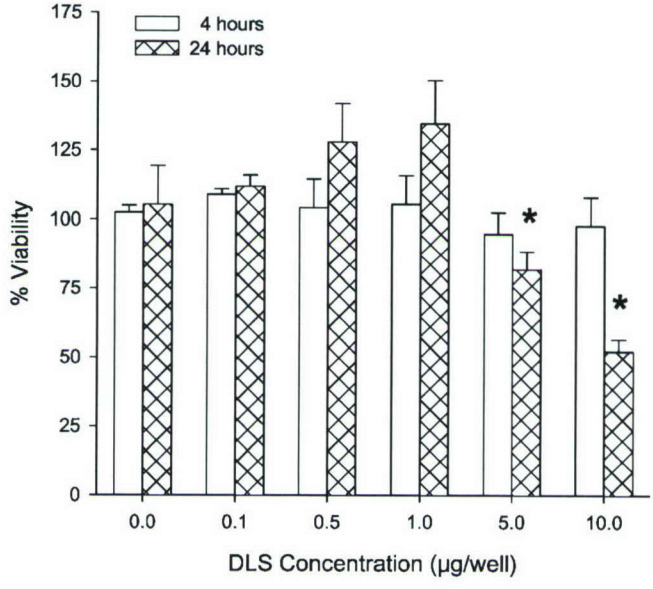

Figure 2 Effect of DLS on viability of NHEK. NHEK were treated with DLS for 4 hours and 24 hours. The drug was removed and viability was determined with the MTS-PMS reagent as in Materials and Methods. Bars indicate mean values ± SD from 3 separate experiments. Data were analyzed by *t*-test and the significance level was $p < 0.05$ (*) compared with nontreated controls.

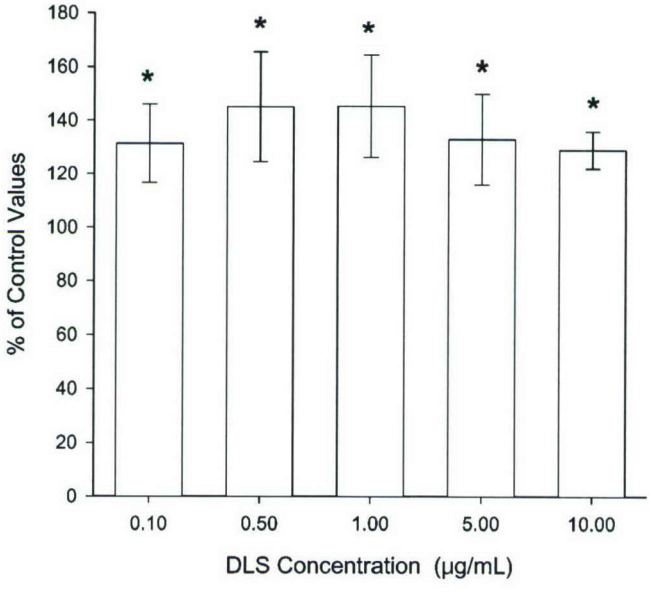

Figure 3 The effect of 4 hours DLS pretreatment on GST levels in NHEK. NHEK were pretreated for 4 hours with the indicated concentrations of DLS. The drug was removed by washing and the cells were processed as described in Materials and Methods. Bars indicate mean values ± SEM from 3 separate experiments. Data was analyzed by *t*-test and the significance level was $p < 0.05$ (*) compared with untreated controls.

times (24 and 48 hours), NHEK exhibit a decrease in GST levels at the higher DLS concentrations. However, the lower concentrations of DLS appear to have no effect on GST levels over 48 hours incubation, since levels in DLS-pretreated NHEK are no different from controls. The optimal DLS concentrations appeared to be in the range of 0.5–1.0 μg/mL for GST induction and were used as pretreatment conditions for cytoprotection against sulfur mustard challenge.

The potential toxicity of this drug at 4 and 24 hours was investigated by incubating NHEK with various concentrations of DLS for these times. The drug was removed by washing, cells were replenished with fresh KGM, and viability was then measured 4 hours later with the MTS-PMS assay. Figure 2 shows that at 4 hours, pretreatment with DLS does not appear to cause any cytotoxicity to NHEK when compared to non-pretreated controls. However, NHEK pretreated with DLS for 24 hours show some cytotoxicity at the higher concentrations of the drug (5 and 10 μg/mL). Therefore, a 4 hour pretreatment time was selected for subsequent studies since the drug was nontoxic and GST levels reached a maximum at this timepoint. Subsequent exposure of NHEK to SM could also be more conveniently performed on the same day.

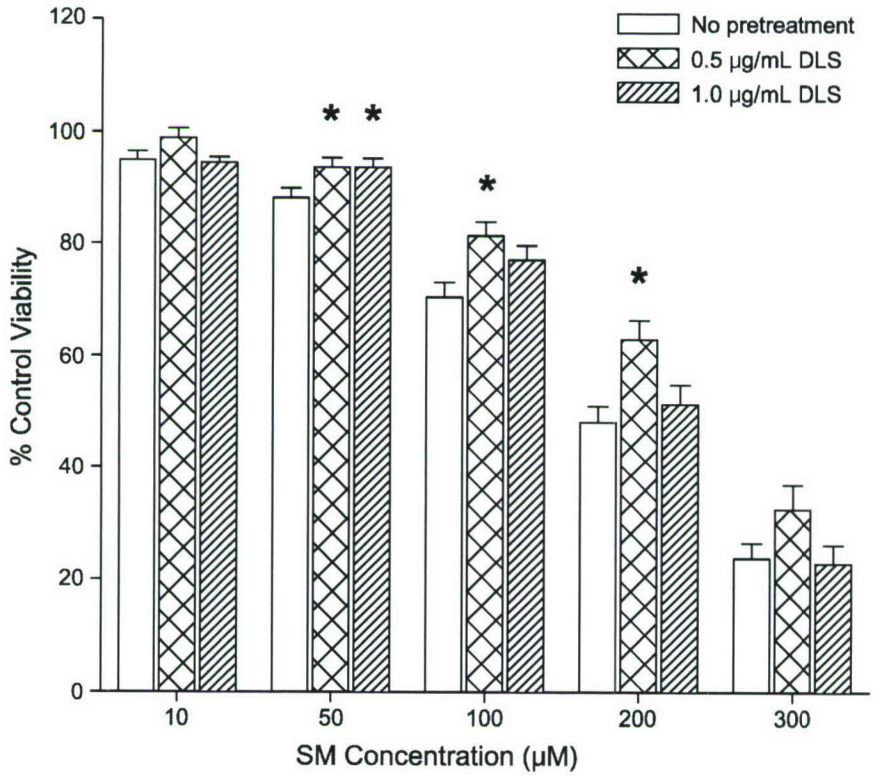

Figure 4 Effect on SM cytotoxicity by DLS pretreatment. The NHEK were pretreated for 4 hours with DLS. The drug was removed by washing; cells were replenished with KGM, and then exposed to SM. Viabilities were determined 24 hours later by the MTS-PMS assay as in Materials and Methods. Bars indicate mean values ± SEM from 3 separate experiments. Data was analyzed by *t*-test; significance level was $p < 0.05$ (*) compared with nonpretreated, non-SM exposed controls.

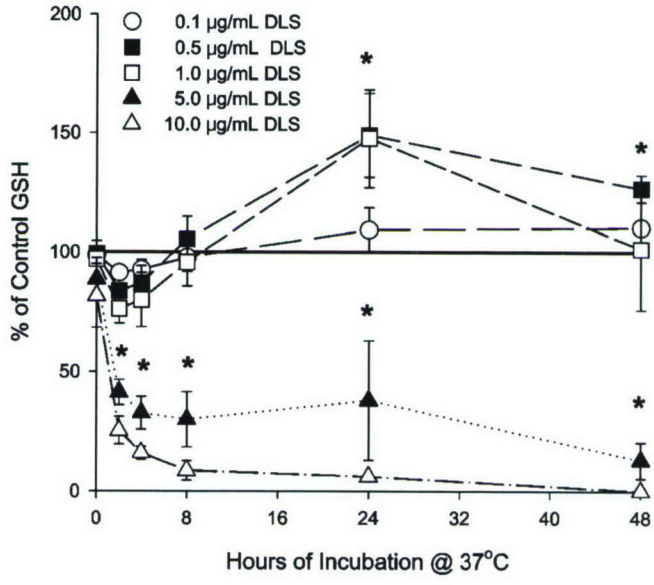

Figure 5 Effect of various DLS concentrations on GSH levels in NHEK. The NHEK were incubated with 0.1–10 µg DLS /mL for up to 48 hours. Culture plates were withdrawn at the indicated times and the drug removed by washing. Cells were frozen at − 80°C and processed for GSH as in Materials and Methods. Bars indicate mean values ± SEM from 3 separate experiments. Data were analyzed by *t*-test and the significance level was $p < 0.05$ (*) compared with nonpretreated controls.

Figure 3 demonstrates the effect of these DLS concentrations on GST levels at 4 hours in more detail. The statistically significant increase in GST appeared at this time with all DLS concentrations studied. However, optimal concentrations of DLS again appeared to be at 0.5 and 1.0 µg/mL, and all pretreatment regimens used these concentrations.

The NHEK were pretreated for 4 hours with these optimal concentrations of DLS, and the drug was then removed by three cycles of washing with saline. Fresh KGM was added to the plates and the cells were challenged with 10–300 µM SM (1 hour at ambient temperature). After the agent had reacted, the plates were replaced in the incubator and incubation continued for another 24 hours. These viabilities were determined 4 hours later by the MTS-PMS assay as outlined in the Materials and Methods section. Viabilities of the pretreated NHEK that had been exposed to SM are expressed as a percentage of the nonpretreated, non-SM exposed control NHEK in Fig. 4. The DLS appeared to have a limited protective effect against exposure of NHEK to 50 µM SM, since there was an approximate 5% increase in viabilities with both DLS pretreatments. At 100 µM SM, the 0.5 ug/mL DLS pretreatment resulted in an 11% increase in viability, while the 1.0 ug/mL pretreatment showed no significant increase in viability. At 200 µM SM, pretreatment with 0.5 ug/mL DLS resulted in a 15% increase in viability, whereas the 1.0 µg/mL DLS pretreatment had no effect on the cytotoxic effect of SM. At the 300 µM concentration of SM, neither DLS pretreatment had any statistically significant protective effect.

Glutathione is known to have a protective effect against sulfur mustard (Fig. 5), so the levels of this tripeptide were also analyzed as a function of the DLS pretreatment. As shown in Fig. 5, pretreatment with the lower concentrations of DLS has no effect on GSH levels for up to 8 hours, but there is an immediate decrease in GSH levels with 5.0 and 10.0 ug/mL DLS. The protective effect of DLS pretreatment at 4 hours cannot be due to the increased levels of GSH. At 24 hours, there appears to be a 50% increase in GSH levels as noted earlier with N-acetyl cysteine (8) and L-Oxothiazolidine 4-carboxylate (9).

CONCLUSIONS

Electrophilic alkylating agents such as SM are known to react extensively with nucleophilic sulfur-containing compounds such as GSH. Increasing intracellular GSH levels through the use of N-acetyl cysteine (8), L-Oxothiazolidine 4-carboxylate (10) and ethacrynic acid (11) pretreatments have shown limited protection against SM.

Another possible protective mechanism against SM may involve formation of a conjugate between GSH and SM by an enzyme-catalyzed process. This mechanism may confer an additional measure of protection. Enzymes that may participate in these types of activities are known as Phase II detoxication enzymes, and specific enzymes such as GST may accomplish this conjugate formation with SM.

The GST can be modified by certain chemicals that either inhibit or induce the formation of these enzymes. The DLS is a selective inducer of Phase II detoxication enzymes such as GST and is known to have anticarcinogenic properties. This compound occurs naturally in broccoli and other vegetables (4).

The role of these enzymes in SM cytotoxicity has not been investigated since SM forms a cyclic sulfonium ion that reacts rapidly with cellular constituents and has a very short half-life. If Phase II detoxication enzyme levels could be modulated, this process may affect the amount of SM that reaches important cellular targets by scavenging the reactive sulfonium species and creating an effective intracellular dose reduction.

The pretreatment of NHEK with DLS for 4 hours has a modest effect ($\sim$40%) on increasing intracellular levels of GST. After the drug has been removed by washing, cells that received fresh media and were exposed to SM appeared to be resistant to the lower concentrations of SM. However, pretreated cells were overwhelmed by 300 μM SM, and the protective effect disappeared since there were no significant differences in cytotoxicity between the pretreated and the non-pretreated NHEK.

Early pretreatment with GST-enhancing drugs may provide a measure of protection against SM through the formation of these putative conjugates, but these conjugates should be quantitated through mass spectrometry or other suitable analytical methods. More efficient compounds that can increase GST levels in target cells may be helpful in designing a medical pretreatment countermeasure against SM. Recently, chemical compounds such as triterpenoids have shown great efficacy in inducing some of the Phase II detoxication enzymes and blocking inflammation in mouse macrophages (12). These compounds may have some utility in protection against the SM injury.

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